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14. ABSTRACT
The primary goal of this collaborative research project is to develop next generation engineered nerve guide conduits (NGCs) with aligned nanofibers and favorable release kinetics of neurotrophic factors to help improve surgical outcomes after injuries involving peripheral nerves.
In the second year of the work, we further improved the gradient loading of the neurotrophic factor GDNF (Glial cell derived neurotrophic factor) and tested various combinations of NGCs in a rat sciatic nerve regeneration model. We have also started the IACUC application for the large animal validation study using the peroneal nerve repair in dogs.

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Introduction:
Peripheral nerve injury is a common complication of complex tissue trauma and often results in significant disability in war injuries. Regeneration of peripheral nerves is often incomplete and in complex war injuries donor nerves are difficult to find for nerve repair. Nerve guide conduits (NGCs) made of biodegradable materials offer a potential solution to this problem. Based on our previous accomplishments in developing a nanofiber containing NGCs, the primary goal of this collaborative research project is to develop new nanofiber NGCs with improved nanofiber guidance cue and modulated trophic factor delivery capabilities that promise faster nerve regeneration and better functional recovery.

Body:

Statement of Work (Proposed Tasks)

The goals of Year 2 included optimization of nanofiber nerve guide design to provide i) contact guidance cue (nanofiber diameter, degradation rate, and fiber density/distribution) and ii) modulated neurotrophic factor delivery (factor choice, concentration range and gradient configuration) for regenerating axons and Schwann cells, and assessing the effects of each modification in a rat model of nerve regeneration.

These goals were outlined in the original Statement of Work as:

Task 2: To assess nerve regeneration rate and functional recovery in a rat model of nerve repair, and to optimize nerve guide configurations

2a. To manufacture of nanofiber nerve guides of without neurotrophic factor loading
2b. To evaluate the effect of nanofiber diameter and degradation rate on nerve regeneration in the rat sciatic model
2c. To manufacture nanofiber nerve guides with optimum neurotrophic factor loading
2d. To evaluate the effect of different neurotrophic factors on nerve regeneration in the rat sciatic model
2e. To prepare and obtain regulatory approval for the dog studies

Progress

Specific experiments are underway to complete these tasks before we initiate the canine study. Significant progresses have been made on optimizing the nanofiber-mediated contact guidance effect and fine-tuning the spatial and temporal presentation of neurotrophic factors in the new generation of nerve guidance conduits (NGCs) (Fig. 1).

Tasks 2a-2d were carried out as outlined below, but unfortunately we have not been able to accomplish all of the goals due to some technical hurdles encountered during the development of gradient neurotrophic factor delivery in the nanofiber nerve conduits. These technical hurdles and our approaches to solving them are outlined in the report below.

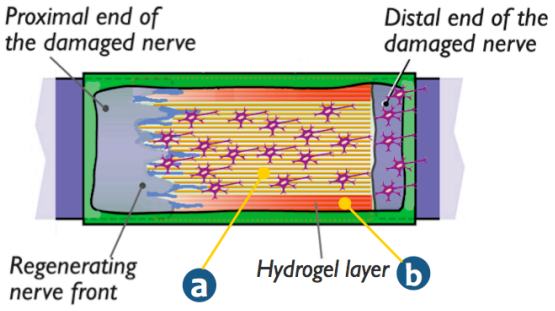

Fig. 1. Design of the nanofiber nerve guides incorporating the nanofiber guidance cues (a) and neurotrophic factor gradient loaded in the hydrogel layer (b) between the outer membrane and nanofibers.

Optimizing Nanofiber Guidance (tasks 2a-2d)

The progress made in this section refers to tasks 2a and 2b (Groups 1-5) and 2c and 2d (Groups 6-19)

We have streamlined the methods for constructing nanofiber conduits and are able to produce nanofiber NGCs in large quantities for *in vivo* studies. The specific parameters evaluated in tasks 2a and 2b are outlined in the table 1 below.

Table 1

Tasks	Groups	# of rats	Fiber distribution	Hydrogel sheet/spacer	Aligned fiber density	Fiber diameter (nm)	GDNF loading (ng/tube)
Task 2a-b	1	8	Spiral	Gelatin	None	N/A	0
	2	8	Single	N/A	Medium	760	0
	3	8	Spiral	Gelatin	Medium	760	0
	4	8	Spiral	Gelatin	High	760	0
	5	8	Spiral	Gelatin	Low	760	0
Task 2c-d	6	8	Spiral	Gelatin	Medium	760	20
	7	8	Spiral	Gelatin	Medium	760	200
	8	8	Spiral	Gelatin	Medium	760	2000

The groups for the first tier of animal studies (Tasks 2a and 2b) were designed to test whether the spiral design, with its increased area of aligned nanofibers, would improve regeneration as compared with the older NGC design wherein the aligned fibers were only in a single layer on the inner luminal wall of the conduit (Fiber distribution column).

This group of animals also examined whether there was any dose response to the nanofibers, by varying the density of nanofibers per unit area of conduit by electrospinning for different lengths of time. The fibers were spun to a density of 0, 1×, 2×, and 3×, relative to one another (Aligned fiber density column).

As outlined below, we are currently examining the effect of nanofiber diameter (Fiber diameter column). In the animals tested so far we kept the nanofiber diameter to the same size as in our original design (760 nm) but want to find out if the nanofiber diameter has a significant effect in vivo.

The first 5 groups of animals that form majority of the tasks 2a and 2b had the surgical repairs done and tissues have been harvested, sectioned, and stained. The soleus and gastrocnemius muscles have been harvested and weighed. We are still performing the imaging and image analysis on the sectioned NGCs, to determine the regenerating nerve area, total number of myelinated axons, total number of unmyelinated axons, and G-ratio.

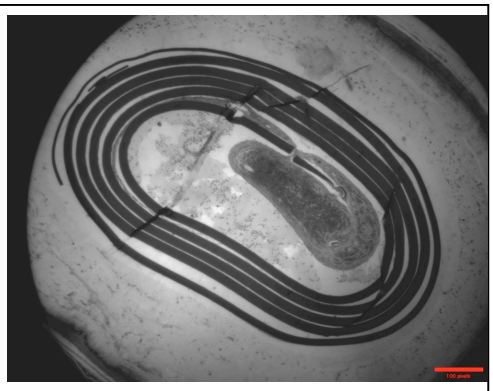

Figure 2. Micrograph of sectioned and stained Group 3 conduit, after 8 weeks in vivo. Slice taken from section of conduit 5-8mm away from proximal end of conduit.

While the full analysis of the first 5 groups is still in progress, we have observed several trends. The control groups with no aligned fibers (Group 1), or the single layer of aligned fibers (Group 5) showed poor regeneration in almost every rat. The rats in the groups with the spiral design (Figure 2) with aligned nanofibers showed better regeneration than most of the rats in the single-layer and non-fiber designs, but there was a great deal of variation from rat to rat within the groups. There was not yet any

obvious trend between the groups with higher densities (2× and 3× fiber densities) of aligned nanofibers (Groups 3 and 4). The group 2 was the original design of nerve conduits without the spiral design and had nerve growth of intermediate numbers.

Although the results were promising for increased axonal regeneration through them, the initial groups have shown some unexpected problems with the spiral NGC design. The gelatin-hydrogel layer degraded much slower than anticipated, with the gelatin films supporting the nanofibers still intact after 8 weeks *in vivo*. At such a late time, the films may be hindering regeneration by slowing nutrient diffusion and blocking the nerve tissue growth, as the gelatin-hydrogel sheets compacted into one another against the outer wall of the conduit (Fig. 2). This may have been caused by hoop stress experienced by the films due to the high crosslinking density.

With these issues for the gelatin sheets, we have developed an alternative approach to the hydrogel layer for the spiral design. Many hydrogel options were initially unfeasible, since we coated the films with nanofibers by electrospinning directly upon them, requiring the films to be sufficiently strong to not break when rotated at over 1000 rpm. We have altered the conduit preparation protocol and prepared fibrin- or collagen-based hydrogel-nanofiber sheets with much lower crosslinking density for making the spiral nerve guidance conduits. We used the diluted Tisseel (clinically used tissue adhesive) as the source of fibrin gel. By replacing the gelatin layer with fibrin hydrogel, the conduit design has been improved in many ways. The spiral layers show no propensity to compact against the outer walls, making all of the aligned nanofibers available to interact with the regenerating axons (Fig. 3). The fibrin-based hydrogel should also degrade over the duration of nerve regeneration (2 to 3 months). The fibrin or collagen concentration is also set so that the Schwann cells and axons should be able to migrate through the hydrogel layer itself, in addition to the space between the nanofiber layers. The literature shows robust migration is possible in hydrogels made from Tisseel diluted to 25% of its original concentration. We have also confirmed that cultured Schwann cells were capable to migrating into fibrin hydrogel along the embedded nanofibers. In addition, varying the thickness of the fibrin layer can control the spacing between the layers.

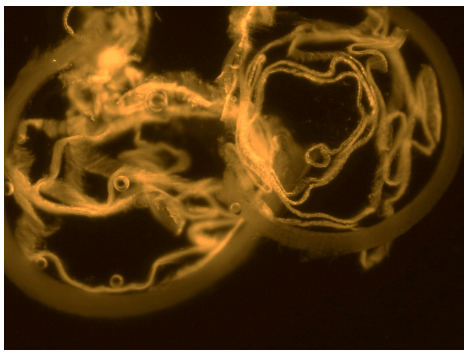

Figure 3. Fluorescence microscopy image of a spiral NGC with collagen as hydrogel, in place of gelatin. The aligned nanofibers have been doped with Rhodamine B to aid visualization. The conduit was sliced with a microtome, and two slices are present in the image with part of the conduit wall missing.

The groups 6-8 included GDNF as the primary variable and were designed to show whether there was any optimal loading level of GDNF within the conduit, with 20 ng, 200 ng, or 2 μg of GDNF per conduit. These animals had their surgical repairs done and evaluated for functional recovery through electrophysiological analysis and showed optimum outgrowth with two of the higher concentrations of GDNF. The NGCs from these groups have been harvested and are still undergoing sectioning and staining for morphological evaluation of axonal regeneration.

Ongoing in vivo studies (Table 2)

Due to these additional trouble-shooting efforts, there is a significant delay in completing the remaining animal studies. There are a few remaining groups need to be tested in rats, to further optimize the hydrogel composition, nanofiber size, and nanofiber composition/degradation rate. In particular, we will verify that the fibrin-based hydrogel spiral will be more effective than the previous gelatin-based spiral. We will also determine whether fibrin is better than collagen as the hydrogel component to support nanofiber distribution within the conduits. Next, we will use the electrospinning parameters optimized over the last year to spin larger and smaller fibers (1200 nm and 400 nm, respectively) to compare with the 760 nm fibers used to date. Finally, we will also determine whether degradable fibers could aid regeneration and overcome the “oasis effect” we have seen on our

previous studies, wherein the favorable microenvironment within the conduit itself prevents proper axonal re-innervation of the distal nerve stump. We will use the result of our previous *in vivo* degradation study (Fig. 4) to choose the composition of the nanofibers, namely 90%gelatin/10%PCL to produce nanofibers that will degrade away by the time the regenerating nerve reaches the distal end of the conduit, freeing it to enter the distal stump.

Another issue that has been slowing the in vivo research progress has been the manufacturing process itself. Previously each conduit needed 2 separate, individual spinning steps to create the outer wall. We have now switched to prefabricating the outer wall, which allows us to spin the outer wall for several conduits concurrently, while also allowing us to strengthen the wall through heat-treatment. Secondly, the gelatin films were very easy to tear at several steps of the process. The fibrin-based hydrogel replacement requires much less handing, and thus fewer rejected samples late in the production cycle. This has greatly improved our production capacity

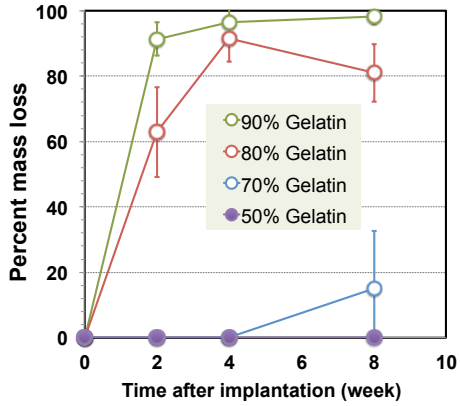

Figure 4. Effect of nanofiber composition on their *in vivo* degradation rate. Mass loss of nanofiber mesh samples were recorded at different time points after implantation in tibial muscles. We successfully made fibers that degraded over the desired timescale of a weeks-to-months by spinning a blend of PCL and gelatin. The nanofibers were then gently cross-linked by being exposed to the vapor phase of a 0.8% glutaraldehyde-in-ethanol mixture at room temperature for 2 days.

Table 2

Tasks	Groups	# of rats	Fiber distribution	Hydrogel sheet/spacer	Aligned fiber density	Fiber diameter (nm)	Nanofiber degradation rate	GDNF loading (ng/tube)
Tasks 2a-d	9	8	Spiral	Tisseel glue (25%)	Medium	760	Low	200
	10	8	Single	Collagen (4 mg/ml)	Medium	760	Low	200
	11	8	Spiral	Tisseel glue (25%)	Medium	760	High	200
	12	8	Spiral	Tisseel glue (25%)	Medium	400	Low	200
	13	8	Spiral	Tisseel glue (25%)	Medium	1200	Low	200

Neurotrophic factor gradient delivery for migration guidance of Schwann cells and regenerating axons (part of tasks 2c and 2d)

One of the critical elements of our tasks 2c and 2d was to develop a reliable and optimum method of delivery of the neurotrophic factors.

Over the past two years, we have been developing a hydrogel-based neurotrophic factor gradient generation platform that is suitable for *in vitro* screening and for incorporating into a nerve guide to repair a damaged nerve *in vivo*. **This is the first such a neurotrophic factor gradient generation method for generating continuous gradient over a large distance (multi-centimeter range) in a convenient and scalable fashion.** We now have developed a protocol to incorporate this growth factor-loaded hydrogel into our nanofiber nerve guides.

In the last year, we have set up a system to evaluate the effect of neurotrophic factor gradient on neural cell migration. Initial studies aimed to explore the effect of GDNF gradients with varied concentration ranges on the directed migration of immortalized human Schwann cells by seeding the cells over gradient-containing hydrogels coated with laminin and fibronectin. These studies resulted in limited overall cell migration and limited directional guidance, largely due to the fact that the hydrogel surface does not provide sufficient cell adhesion for Schwann cell migration. We have modified the experimental setup and incorporated aligned nanofibers over the gradient hydrogel to provide a suitable adhesive substrate for neural cells (Fig. 5a, b).

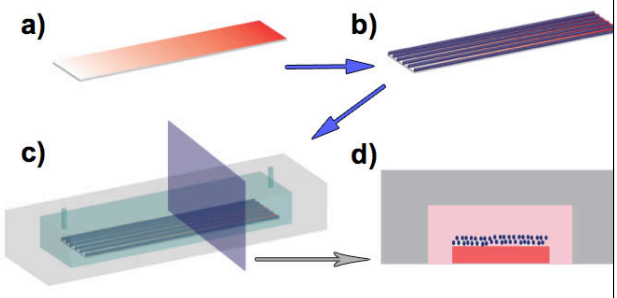

Figure 5. Construction of "limited height" *in vitro* cell migration chamber for measuring cell migration on neurotrophic factor gradient hydrogels and aligned nanofibers. First, gradient hydrogels with controllable gradient characteristics are generated (a). Aligned nanofibers are placed over the hydrogel (b). PDMS channel placed over hydrogel construct and cells are injected into chamber (c). (d) Cross-section of chamber.

On aligned nanofibers with the hydrogel, cell adhesion and migration was markedly improved but directional guidance remained limited (data not shown). We hypothesized that the limited directional guidance was a result of GDNF released from the gradient hydrogel quickly equilibrating within the cell media, thus preventing the cells from sensing significant GDNF gradient and limiting the directional guidance providing by the GDNF released. We then reduced the volume of the cell culture chamber by limiting of the vertical height of the media. With this smaller cell growth volume, which is in fact closer to that of the nerve guides for in vivo applications, the GDNF gradient can be maintained in the horizontal direction for a significantly longer period of time, and thus the biochemical guidance cue would be maintained and greater directional cell migration would be observed. To limit the cell media height, we developed PDMS molds containing a single rectangular channel with 300-µm height, which would be placed over cells seeded on the gradient/nanofiber hydrogels (Fig. 5c, d).

This improved cell culture platform showed significant improvement in cell migration guidance (Fig. 6). We have begun testing the effects of gradients of GDNF and NGF with varied concentration ranges in order to elicit which growth factor and what concentration range of gradient is most effective for directing the migration of Schwann cells. Based on our preliminary results, cells exposed to GDNF or NGF gradients exhibited highly linear migration that was likely due to the contact guidance provided by aligned nanofibers. Both neurotrophic factors demonstrated the ability to promote cell migration towards the high concentration of the gradient, but differences appear to be evident in how the two neurotrophic factors affect the speed and persistence of directional migration. GDNF appeared to provide greater directional preferentiality with a greater degree of cells migrating toward the high concentration compared to those exposed to the

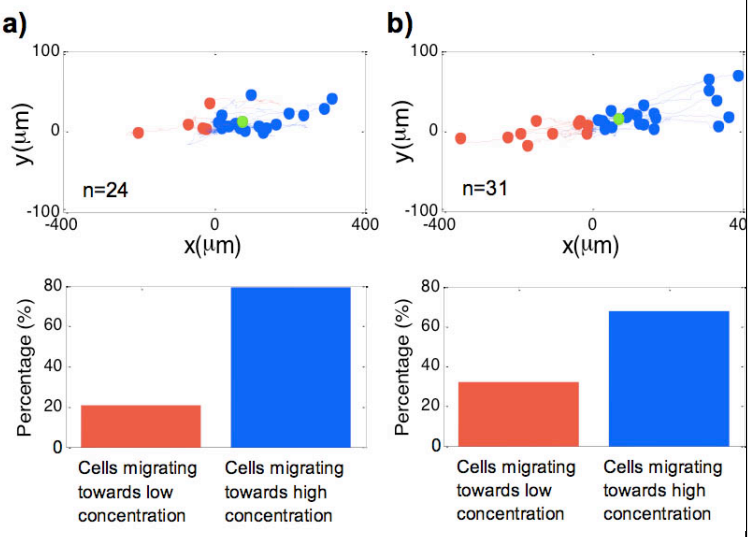

Figure 6. Migration of immortalized Schwann cells on gradient/nanofiber hydrogels with a) 0–10 µg/mL GDNF linear gradient (n = 24), and b) 0–1 µg/mL NGF linear gradient (n = 31). Dots signify final position of each cell relative to its point of origin. Blue dots represent cells which have net migration towards the high concentration of the gradient, whereas red dots represent those

NGF gradient. In contrast, NGF promoted cells to migrate further distances over the same period of time. Further analysis also demonstrated that cells exposed to the NGF gradient maintained their direction of migration (i.e. greater persistence) better than those exposed to the GDNF gradient, which exhibited higher migration velocity but more migratory oscillations. However, it should be noted that these results might be attributed to the differences between the concentration ranges used for both growth factors.

Currently, we are incorporating these optimized gradient configurations (combination, gradient steepness, and concentration range) into nanofiber conduits, and we will test the effectiveness of these trophic factor gradients in the rat model of sciatic nerve repair. These groups (14-19) will be the last part of the tasks 2c and 2d (Table 3) and will allow us to move ahead with the best conduit design in the large animal model of peripheral nerve repair.

Table 3

Tasks	Groups	# of rats	Fiber distribution	Hydrogel sheet/spacer	Aligned fiber density	Fiber diameter (nm)	Nanofiber degradation rate	GDNF gradient release	NGF gradient release
Tasks 2c-d	14	8	Spiral	Tisseel glue (25%)	Medium	760	Low	Slow	-
	15	8	Single	Tisseel glue (25%)	Medium	760	Low	Fast	-
	16	8	Spiral	Tisseel glue (25%)	Medium	760	Low	-	Slow
	17	8	Spiral	Tisseel glue (25%)	Medium	760	Low	-	Fast
	18	8	Spiral	Tisseel glue (25%)	Medium	760	Low	Slow	Slow
	19	8	Spiral	Tisseel glue (25%)	Medium	760	Low	Fast	Fast

Regulatory approval for the dog studies (Task 2e)

The protocol has been developed and is being submitted to the institutional animal care and use committee (IACUC). Once approval is obtained we will submit it for ACURO approval.

We are currently preparing 4 manuscripts to document the progress we have made in the last 2 years. The titles of these manuscripts in preparation have been removed from the “Reportable Outcomes” section of this report.

Key Research Accomplishments:
Refinement of the nanofiber NGCs:

- Increased nanofiber surface area
- Fine-tuning of nanofiber degradation rate
- Gradient loading of neurotrophic factors

Validation studies in the rat sciatic nerve regeneration model

- Tested the role of increased nanofiber surface area (spiral design)
- Tested the role of gelatin hydrogel with GDNF loading
- Tested the role of nanofiber density

Reportable Outcomes

Manuscripts Published
Krick K, Tammia M, Martin R, Höke A, Mao HQ. Signaling cue presentation and cell delivery to promote nerve regeneration. Current Opinions in Biotechnology, 22(5): 741-746 (2011).

Scientific Presentations

Martin R, Mi R, Mullen B, Ginn A, Höke A* and Mao HQ*. Optimization of nanofiber configuration in nerve guidance conduits, Poster Presentation at the Society for Neuroscience Annual Meeting, New Orleans, October 2012

Krick KD, Khademhosseini A, Höke A* and Mao HQ*. Neurotrophic factor gradient generation for directional peripheral nerve growth guidance and regeneration, Poster Presentation at the Society for Neuroscience Annual Meeting, New Orleans, October 2012

Conclusion:
We have improved upon on the original design of the nanofiber NGCs and have been carrying out the in vivo optimization studies in the rat model of nerve regeneration. In the next year, we will complete the rat studies and carry out the validation study in the dog peroneal nerve regeneration model.

References:
None

Appendices:
None